

## ORIGINAL PAPER



# Different patterns of p16<sup>INK4a</sup> immunohistochemical expression and their biological implications in laryngeal squamous cell carcinoma

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## Abstract

**Introduction:** p16<sup>INK4a</sup> immunohistochemistry (IHC) is widely used to facilitate the diagnosis of human papillomavirus (HPV)-associated neoplasia, when  $\geq 70\%$  of cells show strong nuclear and cytoplasmic positivity. In this study, we aim to compare partial expression patterns that do not fulfill the above criteria and seek biological implications in laryngeal squamous cell carcinoma (LSCC). **Materials and Methods:** p16<sup>INK4a</sup> IHC staining was conducted on representative sections of archived tissue from 88 LSCCs. Immunoreactivity was described based on four parameters: intracellular localization of immunostaining, intensity of immunostaining, distribution pattern and percentage of positive cells. **Results:** Six patterns of p16<sup>INK4a</sup> immunoreactivity were observed and defined as: strong diffuse (strong immunostaining, expression in cytoplasm and nucleus in  $>70\%$  of tumor cells), weak diffuse (moderate or weak immunostaining, expression in cytoplasm in  $>70\%$  of tumor cells), marginal (strong cytoplasmic immunostaining, limited to the periphery of tumor islets), strong scattered (strong immunostaining, expression in cytoplasm and nucleus in  $<50\%$  of tumor cells), weak scattered (moderate or weak immunostaining, expression in cytoplasm in  $<50\%$  of tumor cells), negative (no expression). The pN stage of the patients was associated with p16<sup>INK4a</sup> immunoreactivity patterns, the marginal pattern was only found in the pN0–Nx stages, while the weak diffuse pattern was more frequently observed in pN2–N3 stages. **Conclusions:** Partial immunostaining with architecturally distinct p16<sup>INK4a</sup> immunoreactivity patterns may prove significant in stratifying characteristic clinicopathological subgroups among LSCC. Our observations may support the hypothesis that p16<sup>INK4a</sup> has different roles in different subcellular locations, with tumorigenic molecular pathways unrelated to HPV infection.

**Keywords:** larynx, squamous cell carcinoma, p16, immunohistochemistry.

## Introduction

Laryngeal carcinomas are the second most common respiratory tract cancers after lung cancer, thus representing an important global health burden [1]. A marked variation in frequency, both geographical and gender wise, has been noted, squamous cell carcinomas (SCCs) accounting for most histological types [2]. The main risk factors are represented by tobacco and alcohol use. The role of the human papillomavirus (HPV) in the pathogenesis of laryngeal squamous cell carcinoma (LSCC) is still controversial, but research in this area has contributed to a more thorough study of p16<sup>INK4a</sup>, whose immunohistochemical expression is used as a surrogate marker for the presence of HPV in cervical and oropharyngeal carcinoma [3–5].

The cell cycle of resting cells is strictly managed through various checkpoints by a set of regulatory proteins. One example is the regulation of G1-to-S progression by two classes of cyclin and cyclin-dependent kinase (CDK) complexes: cyclin Ds–CDK4/6 and cyclin Es–CDK2.

These complexes inactivate the retinoblastoma protein (pRB) family through phosphorylation and promote the progression towards mitosis [6]. p16<sup>INK4a</sup> is one of the cyclin–CDK inhibitor proteins; it binds directly to CDK4 and CDK6 and blocks phosphorylation of the pRB, maintaining it in a hypo-phosphorylated and growth-suppressive state and inducing a G1 phase cell cycle arrest [7]. Isolated, characterized, and named in the early 1990's, this 16 kDa protein is part of the INK4 class of cell cycle inhibitors; the encoding gene, cyclin-dependent kinase inhibitor 2A (CDKN2A) or multiple tumor suppressor 1 (MTS1), has an 8.5 kb length and is located on chromosome 9p21.3 [8, 9]. Considering the role played by the retinoblastoma tumor suppressor (RB) pathways in blocking inappropriate cellular proliferation, the loss of p16<sup>INK4a</sup> and its negative regulator function on the cell cycle is thought to lead to carcinogenesis [10].

The main focus of research for p16<sup>INK4a</sup> immunoreactivity has been the well-established correlation with HPV infection. In the infected cells, the E7 viral oncoprotein functionally inactivates the pRB, and, released from its

negative feedback control, p16<sup>INK4a</sup> becomes upregulated, in an attempt to inhibit uncontrolled cellular replication. This leads to an increase of intracellular levels of p16<sup>INK4a</sup> that can be detected by immunohistochemistry (IHC), rendering this protein an excellent surrogate marker for HPV infections in some settings [11]. In the context of HPV related lesions, there is marked heterogeneity in p16<sup>INK4a</sup> IHC scoring, different researchers defining positivity based on different combinations of parameters like: the percentage of immunostained cells, immunostaining pattern, and immunostaining intensity. Most practices follow the criteria recommended by the lower anogenital squamous terminology (LAST): a positive result should be considered when there is “block-positivity” (defined as continuous, strong nuclear immunostaining, with or without cytoplasmic signal, with extension from the basal cell layer upward for at least one third of the thickness of the epithelium) [12, 13]. In a tumor sample, “block-positivity” is usually translated as strong nuclear ± cytoplasmic positivity in more than 70% of tumoral cells. Judicious interpretation of the results is advisable regarding cases that are not clear-cut positive or negative, since some ambiguous patterns have been shown to harbor HPV [14, 15].

Various other stressors lead to an aberrant expression of CDKN2A. As a tumor suppressor protein, p16<sup>INK4a</sup> has been linked to senescence and multiple tumors, like lymphoma, melanoma, odontogenic tumors, pancreatic adenocarcinoma, non-small cell lung cancer, gastrointestinal carcinoma, and prostate cancer, to name just a few [16–18]. Not surprisingly, various models of IHC expression have come to the attention of researchers.

### Aim

In this study, we aimed to assess the IHC expression patterns of p16<sup>INK4a</sup> in the setting of LSCC. We focused on partial expression patterns that did not meet the “block-positivity” criteria. Possible biological implications were analyzed using clinicopathological variables and patient outcomes.

## Materials and Methods

### Tissue specimens

The material was comprised of formalin-fixed paraffin-embedded (FFPE) tissue samples of laryngeal carcinomas collected in 2009 and 2010. Following a protocol approved by the Medical Ethics Committee of Iuliu Hațieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania (Approval No. 177/10.05.2016), the Emergency County Hospital Pathology Department’s Database was searched for consecutive patients surgically treated at the Department of Otolaryngology. Tumor samples were selected based on the following criteria: origin in the larynx, sufficient FFPE material left, and accessible clinical and histopathological information. Detailed clinical and pathological data, including information on smoking history, alcohol consumption, treatment, and outcomes were obtained from medical records and from the Institutional Cancer Registry of the Prof. Dr. Ion Chiricuță Oncology Institute, Cluj-Napoca. Patients who underwent previous treatment for their cancer were excluded. Written informed consent was provided by all patients prior to the surgical procedure.

In addition to the initial evaluation for routine diagnostics, all cases were reviewed by two study pathologists and histologically assessed according to the *World Health Organization (WHO) Classification of Head and Neck Tumours* (4<sup>th</sup> edition) [2] recommendations.

### IHC staining

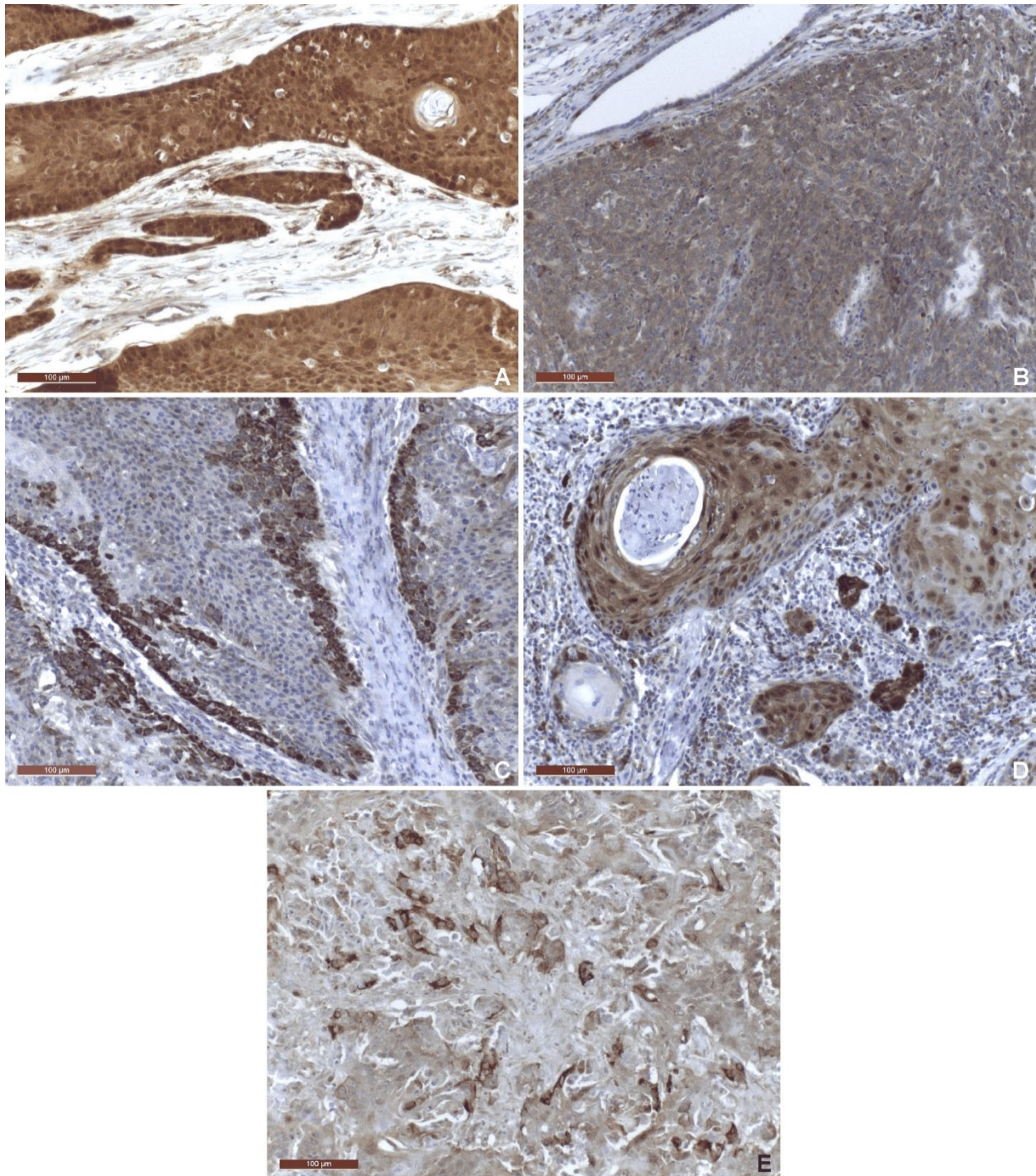
For each case, representative 3 μm sections of archived FFPE tissue from surgical specimens were placed on silanized glass slides (Dako, Glostrup, Denmark), deparaffinized, and rehydrated through a series of xylene and graded alcohols (100%, 95%, and 75%). Antigen retrieval pretreatment was performed with Novocastra™ Epitope Retrieval Solution pH 9 (Leica Biosystems, Newcastle upon Tyne, United Kingdom) for 20 minutes at 100°C. p16<sup>INK4a</sup> IHC staining was conducted using a ready-to-use anti-p16 mouse monoclonal antibody, clone G175-405 (BioGenex, Fremont, CA, USA). Ki67 IHC staining was conducted using anti-Ki67 mouse monoclonal antibody, clone MM1 (Novocastra, Leica Biosystems), 1:100 dilution. Incubation was carried out for one hour at room temperature. Detection involved Super Sensitive™ Polymer Horseradish Peroxidase (HRP) + 3,3'-Diaminobenzidine (DAB) IHC Detection System (BioGenex), used according to the manufacturer’s protocol. DAB was used as chromogen, in order to visualize antigen location sites. Hematoxylin was used for counterstaining. Positive controls consisting of uterine cervix with severe dysplasia (for p16<sup>INK4a</sup> immunostaining) and tonsil (for Ki67 immunostaining) were included routinely.

### Evaluation of IHC expression

Two independent investigators analyzed the expression of the tested protein with the use of a DM750 (Leica Biosystems) light microscope coupled with an ICC50 high definition (HD) camera.

p16<sup>INK4a</sup> immunoreactivity was described following four parameters: intracellular localization of the immunostaining (nuclear, cytoplasmic, both), immunostaining intensity (strong, moderate, mild; positive immunostaining was defined as visual detection of any appreciable shade of DAB beyond the baseline Hematoxylin counterstaining), distribution of positive cell (diffuse – in all layers of tumor islets, limited to the periphery of tumor islets, or scattered as individual cells or as groups of less than 50 contiguous cells demonstrating immunostaining), and the proportion of immunostained tumor cells, semi-quantitatively categorized in increments of 10% by visual estimation. The five most representative 200×-magnification microscopic fields were selected for evaluation.

Based on these parameters, six patterns of p16<sup>INK4a</sup> immunoeexpression were observed and defined as: (i) strong diffuse – strong immunostaining expression in cytoplasm and nucleus in >70% of tumor cells (Figure 1A), (ii) weak diffuse – moderate or weak immunostaining expression in cytoplasm in <70% of tumor cells (Figure 1B), (iii) marginal – strong cytoplasmic immunostaining, limited to the periphery of tumor islets (Figure 1C), (iv) strong scattered – strong immunostaining expression in cytoplasm and nucleus in <50% of tumor cells (Figure 1D), (v) weak scattered – moderate or weak immunostaining expression in cytoplasm in <50% of tumor cells (Figure 1E), and (vi) negative – no expression.



**Figure 1** – Representative examples of p16<sup>INK4a</sup> immunohistochemical patterns observed in laryngeal squamous cell carcinoma: strong diffuse (A), weak diffuse (B), marginal (C), strong scattered (D), weak scattered (E). Figures were captured at 200× magnification.

Evaluation of Ki67 immunoreactivity reported the proliferation index, represented by the proportion of tumor cells with nuclear immunostaining in increments of 10%, assessed on 10 400×-magnification microscopic fields, each field containing about 1000 cells.

#### Statistical analysis

Statistical analyses were performed using *R* with R Commander version 3.6.2. p16<sup>INK4a</sup> immunorexpression was correlated with baseline clinical and pathological characteristics of the patients, as well as their immuno-

profile. Fisher's exact test of independence was used to analyze categorical variables. Differences between groups were assessed using Mann–Whitney *U*-test or Kruskal–Wallis test for continuous variables, followed, if significant, by *post-hoc* analysis for all pairwise comparisons. Shapiro–Wilk test was used to test the assumption of normality.

Overall survival (OS) and disease-free survival (DFS) were calculated using Kaplan–Meier method; OS was measured from date of initial diagnosis to date of death or last follow-up; DFS was calculated from the date of initial diagnosis to that of disease progression, defined as

cancer recurrence, metastasis, or cancer-related death. Log-rank test was used for analyses evaluating survival by p16<sup>INK4a</sup> expression.

*p*-value was considered statistically significant if <0.05. When multiple pairwise comparisons were necessary, Bonferroni correction of the *p*-values was used in order to control the familywise error rate.

## Results

### Clinical and pathological features

A total of 88 patients with sufficient tumor samples for p16<sup>INK4a</sup> immunostaining were identified and included in this study. All patients were males, with the age range between 45 and 76. Most of the patients had smoking histories, with only nine reported as never smoker. Almost three quarters of the patients admitted to drinking alcohol, habitually or occasionally. Most of the specimens were glottic tumors, less than a third belonging to supraglottic or subglottic subsites. A slight predominance of pT1 stage

was observed when analyzing the distribution of cases according to pT stage. Positive lymph node status was noted in 19 cases, five corresponding to pN1 and 14 to pN2 stage; no lymph node metastases were identified in the remaining 69 cases. For 23 out of the latter 69 cases, the number of evaluated lymph nodes did not allow accurate pN staging (pNx). Most LSCCs exhibited moderate histological differentiation. SCCs exhibited non-conventional histology in seven (7.95%) cases, the histological subtypes observed along with the conventional SCC being verrucous, basaloid, papillary and spindle cell SCC. Evidence of keratinization was seen in 69 out of 88 cases. Lymph vessel invasion was detected in 20 (22.72%) cases, while blood vessel invasion was detected in seven (7.95%) cases. A minority of cases displayed perineural invasion (five out of 88). Approximately 50% of the patients received radiotherapy/chemoradiotherapy after surgery.

Detailed clinical and pathological characteristics of patients can be seen in Table 1.

**Table 1 – Clinicopathological characteristics by p16<sup>INK4a</sup> expression patterns**

Variables	All patients (N=88) n (%)	Strong diffuse (N=3) n (%)	Weak diffuse (N=6) n (%)	Marginal (N=17) n (%)	Strong scattered (N=3) n (%)	Weak scattered (N=31) n (%)	Negative (N=28) n (%)	<i>p</i> -value <sup>a</sup>
<b>Age</b>								0.929
mean±SD [years]	58.5±7.8	58.3±14.9	60.5±9.2	58.2±5.3	56.6±8.3	57.7±7.5	59.2±8.9	
<b>Smoking</b>								0.442
• never smoker	9 (10.3)	1 (33.3)	1 (16.7)	2 (11.8)	0	1 (3.2)	4 (14.3)	
• smoker <sup>b</sup>	79 (89.7)	2 (66.7)	5 (83.3)	15 (88.2)	3 (100)	30 (96.8)	24 (85.7)	
<b>Alcohol use</b>								0.435
• no	25 (28.4)	0	1 (16.7)	5 (29.4)	2 (66.7)	11 (35.5)	6 (21.4)	
• yes	63 (71.6)	3 (100)	5 (83.3)	12 (70.6)	1 (33.3)	20 (64.5)	22 (78.6)	
<b>Anatomical subsite</b>								0.534
• supraglottis	20 (22.7)	0	3 (50)	5 (29.4)	1 (33.3)	6 (19.4)	5 (17.8)	
• glottis	64 (72.7)	3 (100)	3 (50)	10 (58.8)	2 (66.7)	24 (77.4)	22 (78.6)	
• subglottis	4 (5.4)	0	0	2 (11.8)	0	1 (33.2)	1 (3.6)	
<b>T-stage</b>								0.279
• pT1	31 (35.2)	2 (66.7)	1 (16.7)	8 (47)	1 (33.3)	8 (25.8)	11 (39.3)	
• pT2	11 (12.5)	0	2 (33.3)	2 (11.7)	2 (66.7)	2 (6.5)	3 (10.7)	
• pT3	21 (23.9)	0	1 (16.7)	2 (11.7)	0	11 (35.5)	7 (25)	
• pT4	25 (28.4)	1 (33.3)	2 (33.3)	5 (29.4)	0	10 (32.2)	7 (25)	
<b>N-stage</b>								0.004 <sup>c</sup>
• pNx	23 (26.1)	2 (66.7)	1 (16.7)	3 (17.6)	1 (33.3)	7 (22.6)	9 (32.1)	
• pN0	46 (52.3)	1 (33.3)	1 (16.7)	14 (82.4)	2 (66.7)	15 (48.4)	13 (46.4)	
• pN1-N2	19 (21.6)	0	4 (66.6)	0	0	9 (29)	6 (21.5)	
<b>Differentiation</b>								0.073
• well	17 (19.3)	0	0	4 (23.5)	1 (33.3)	4 (12.9)	8 (28.6)	
• moderate	47 (53.4)	3 (100)	3 (100)	10 (58.8)	1 (33.3)	13 (41.9)	17 (60.7)	
• poor	24 (27.3)	0	0	3 (17.7)	1 (33.3)	14 (45.2)	3 (10.7)	
<b>Histological subtype</b>								0.356
• conventional	81 (92)	3 (100)	4 (66.6)	17 (100)	3 (100)	28 (90.4)	26 (92.8)	
• verrucous	1 (1.1)	0	0	0	0	1 (3.2)	0	
• basaloid	2 (2.3)	0	0	0	0	1 (3.2)	1 (3.6)	
• papillary	1 (1.1)	0	1 (16.7)	0	0	0	0	
• spindle cell	3 (3.5)	0	1 (16.7)	0	0	1 (3.2)	1 (3.6)	

Variables	All patients (N=88) n (%)	Strong diffuse (N=3) n (%)	Weak diffuse (N=6) n (%)	Marginal (N=17) n (%)	Strong scattered (N=3) n (%)	Weak scattered (N=31) n (%)	Negative (N=28) n (%)	p-value <sup>a</sup>
<i>Keratinization</i>								0.809
▪ present	69 (78.4)	3 (100)	4 (66.6)	13 (76.5)	3 (100)	23 (74.2)	23 (82.1)	
▪ absent	19 (21.6)	0	2 (33.4)	4 (23.5)	0	8 (25.8)	5 (17.9)	
<i>Lymphovascular invasion</i>								0.905
▪ negative	65 (73.9)	2 (66.7)	5 (83.3)	12 (70.6)	2 (66.7)	22 (71)	22 (78.6)	
▪ positive	23 (26.1)	1 (33.3)	1 (16.7)	5 (29.4)	1 (33.3)	9 (29)	6 (21.4)	
<i>Perineural invasion</i>								0.297
▪ negative	83 (94.3)	3 (100)	6 (100)	16 (94.1)	3 (100)	27	28 (100)	
▪ positive	5 (5.67)	0	0	1 (5.9)	0	4	0	

N, n: No. of cases; SD: Standard deviation. <sup>a</sup>Statistical analysis performed excluding the strong diffuse group; <sup>b</sup>Former or current smoker; <sup>c</sup>Statistical analysis performed excluding the pNx group.

Median follow-up time for the patients was 5.66 years (with a maximum of 7.28 years).

During follow-up, 34 deaths occurred. In the full cohort, the 5-year OS was 63.6% [95% confidence interval (CI): 54.3%–75.5%], and the 5-year DFS was 51.1% (95% CI: 41.7%–62.7%).

### IHC assessment

IHC study showed positivity to p16<sup>INK4a</sup> in 68.18% (n=60) of the samples. Overall, three (3.41%) patients had a strong diffuse pattern of p16<sup>INK4a</sup> immunopositivity, six (6.82%) patients had a weak diffuse immunostaining pattern, 17 (19.32%) patients had a marginal immunostaining pattern, three (3.41%) patients had a strong scattered immunostaining pattern, and 31 (35.23%) patients had a weak scattered immunostaining pattern. p16<sup>INK4a</sup> was negative in the remaining 28 (31.82%) cases.

In order to compare the partial p16<sup>INK4a</sup> immunopositivity patterns that do not meet the classical HPV-related positivity criteria (more than 70% of the cells demonstrate intense nuclear and cytoplasmic positivity) and to analyze their potential biological implications in LSCC, statistical analyses were performed without the strong diffuse group.

Variables such as age, smoking, alcohol consumption, anatomical site, histological subtype, keratinization, lymphovascular and perineural invasion, or pT stage did not show significant differences for p16<sup>INK4a</sup> immunopositivity patterns. However, there was a statistically significant association between the p16<sup>INK4a</sup> immunostaining groups and the pN staging. The marginal pattern was only found in the pN0 stages and cases in which lymph nodes were not harvested due to no clinical suspicion of lymph node metastasis (pNx). To avoid misinterpretation of the real nodal status, further analysis of the relation between p16<sup>INK4a</sup> immunostaining groups and the pN staging disregarded the pNx cases (Table 1).

*Post-hoc* test comparing the group of tumors with marginal immunopositivity pattern to the other immunopositivity patterns, by pN stage, the statistical significance is retained ( $p=0.006$  on Fisher's exact test, new significant  $p$ -value after Bonferroni correction  $<0.01$ ) (Figure 2). Separate analyses of the relationship between each type of pattern according to the pN stage, using the Bonferroni correction, shows that the comparison with statistically significant results was that between marginal and weak diffuse (Table 2).

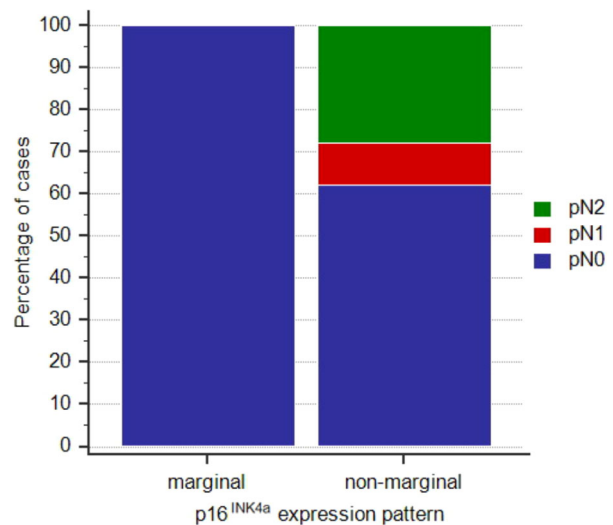


Figure 2 – Distribution of cases with marginal immunopositivity pattern versus other patterns, by pN stage.

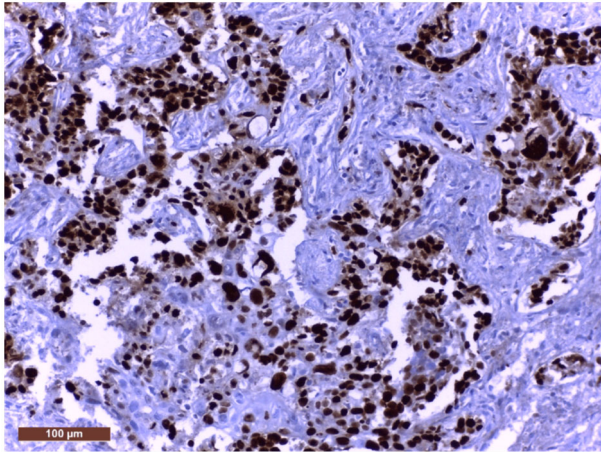
Table 2 – Pairwise comparisons of p16<sup>INK4a</sup> expression patterns by pN stage

Compared patterns	p-value <sup>a</sup>
Marginal vs Negative	0.027
Marginal vs Weak scattered	0.014
Marginal vs Strong scattered	–
Marginal vs Weak diffuse	0.001
Negative vs Weak scattered	0.755
Negative vs Strong scattered	1
Negative vs Weak diffuse	0.122
Strong scattered vs Weak scattered	0.529
Strong scattered vs Weak diffuse	0.142
Weak scattered vs Weak diffuse	0.143

<sup>a</sup>Significant  $p$ -value after Bonferroni correction is  $p<0.005$ .

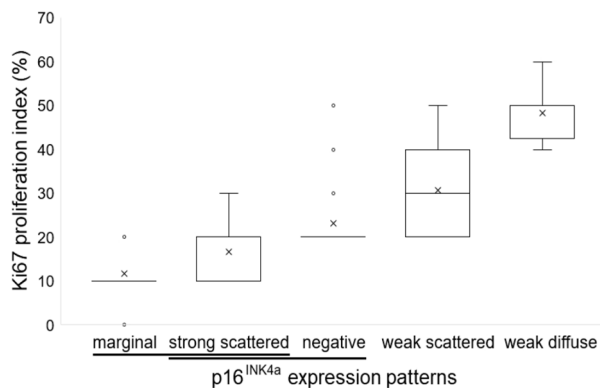
The Ki67 immunostaining showed positivity in 86 out of 88 cases. In well-differentiated tumors, the average Ki67 proliferation index was  $17.05\pm 6.85\%$ . Poorer histological differentiation was associated with a significantly higher Ki67 proliferation index ( $p<0.001$ , Kruskal–Wallis test), with average values of  $24.77\pm 11.51\%$  for moderately differentiated carcinoma and  $31.66\pm 12.74\%$  for poorly differentiated forms (Figure 3). The proportion of Ki67-positive cells was significantly higher in advanced stages ( $p<0.001$ , Kruskal–Wallis test), values rising from  $18.62\pm$

7.89% and 23.63±12.86% for pT1 and pT2 tumors to 27.14±10.55% and 32.08±13.50% for pT3 and pT4 tumors. The same trend was observed regarding pN stages ( $p<0.001$ , Kruskal–Wallis test), with average values of Ki67 proliferation index ranging from 22.66±11.75% in pN0 cases to 37.14±10.69% in pN2 cases. There was no statistically significant difference between average values of Ki67 proliferation index depending on anatomical site, smoking habits, lymphovascular or perineural invasion (all  $p$ -values  $>0.05$  on Kruskal–Wallis, respectively Mann–Whitney  $U$ -test).



**Figure 3** – Ki67 immunostaining in poorly differentiated squamous cell carcinoma. Figure captured at 200× magnification.

When investigating the IHC expression of Ki67 in the described p16<sup>INK4a</sup> immunorepression patterns, the mean values of Ki67 proliferation index were found to be significantly heterogeneous among groups ( $p<0.001$ , Kruskal–Wallis test). The average value of Ki67 proliferation index was lowest in the marginal immunostaining pattern group (11.76±5.28%). Tumors with weak p16<sup>INK4a</sup> IHC expression, like the weak diffuse and the weak scattered immunostaining pattern groups had significantly higher values of the Ki67 proliferation index (48.33±7.52% and 30.64±8.92%) (Figure 4).



**Figure 4** – Box-whisker plot showing distribution of Ki67 proliferation index according to different p16<sup>INK4a</sup> expression patterns. Circles represent outside values; “x” markers represent means. Pairs of means grouped by a horizontal line are not significantly different from each other on post-hoc analysis.

In OS and DSF analyses, p16<sup>INK4a</sup> immunorepression patterns showed no significance, neither in the general

cohort ( $p=0.117$ , respectively  $p=0.094$ , log-rank test), nor in the radiotherapy/chemotherapy group ( $p=0.89$ , respectively  $p=0.186$ , log-rank test).

## Discussions

This study is focused on equivocal p16<sup>INK4a</sup> IHC expression patterns in LSCC. We revealed a significant association between p16<sup>INK4a</sup> immunolabeling and both pN staging and Ki67 proliferation index.

The conventionally accepted criteria for p16<sup>INK4a</sup> IHC positivity (strong, diffuse nuclear ± cytoplasmic immunorepression observed in  $>70\%$  of tumoral cells) implies that all other staining patterns are to be scored negative or regarded as not related to HPV infection. This consensus, however, has been set by studies on oropharyngeal, cervical, and anal carcinomas. p16<sup>INK4a</sup> immunorepression and its distinct architectural morphologies in non-oropharyngeal head and neck squamous cell carcinoma (HNSCC) are yet to be fully evaluated [19].

Although deemed by most studies as nonspecific, we considered the cytoplasmic localization of p16<sup>INK4a</sup> as positive and included it among the described parameters. Ultrastructural studies have suggested it should not be ignored: electron microscopy evidence of its specific cytoplasmic immunolocalization has been published [20], and subcellular fractionation confirmed that the nuclear and cytoplasmic fractions seem to be the same [21]. Moreover, both Zhao *et al.* [22] and Lai *et al.* [23] hypothesized that nuclear and cytoplasmic p16<sup>INK4a</sup> immunorepression have different prognostic implications in HNSCC and respectively, oropharyngeal carcinoma. Possible explanations for the underlying mechanism leading to cytoplasmic accumulation of p16<sup>INK4a</sup> vary from damage of the cytoplasmic–nucleus shuttling in a manner similar to that reported for breast cancer 1 (*BRC1*), to it representing a means of inactivating p16<sup>INK4a</sup> or a defect protein localized to the cytoplasm secondary to mutations of *p16* gene [20, 21].

Different immunolabeling patterns of p16<sup>INK4a</sup> have been studied mainly on HNSCC as a group, most studies performing tests on tumor samples from various anatomical sites, with no studies focused on the larynx, to our knowledge. Variations in histological characteristics of SCC in different anatomical sites might correlate with p16<sup>INK4a</sup> immunorepression patterns; numerous studies have indeed underlined the association of oropharyngeal basaloid or nonkeratinizing SCC (distinct features of HPV+ tumors) with a strong, diffuse p16<sup>INK4a</sup> positivity. Moreover, this correlation has been incorporated in the most recent Tumor, Node, Metastasis (TNM) staging system for HNSCC [24]. But this tight relation between HPV and p16<sup>INK4a</sup> is not found in LSCC, an important percentage of these tumors being HPV-/p16+ or HPV+/p16-, further proof that the protein’s increased expression is not completely specific to pRB-E7 oncoprotein pathway [25].

Our study identified four patterns of p16<sup>INK4a</sup> immunorepression: marginal, weak diffuse, strong scattered, weak scattered, alongside the strong diffuse and negative cases. Similar to previous studies [26, 27], we noted a low frequency of the strong diffuse pattern (conventional positivity) in our cohort. This low proportion of p16<sup>INK4a</sup> positive LSCC cases has been reported even by authors

that chose to use a less stringent cutoff for p16<sup>INK4a</sup> positivity, like 30% [27].

Attempting to correlate p16<sup>INK4a</sup> with cancer immunity, Ryu *et al.* [19] analyzed the architectural patterns of IHC expression in HNSCC, identifying five distinct types of immunostaining: strong, marginal, mosaic, nuclear, and absent. As expected, the strong pattern (conventional positivity) was most frequently noted for the oropharynx, with less than 10% of laryngeal cases showing this type of immunoreexpression. In contrast to our study, the marginal pattern, which was defined as partial staining restricted to tumor margins or tumor buds, without specifying intracellular localization, was not observed in LSCC, but only for oral cavity SCC. This might be explained by the smaller number of LSCC included in the study – 42, as opposed to the 202 oral cavity SCC. Kindred categories were used by Chen *et al.* [28] to describe equivocal p16<sup>INK4a</sup> immunostaining in HNSCC: isolated cells at periphery of nests, faint-diffuse, faint-isolated, and faint-diffuse with patches of strong immunostaining. Chen *et al.*'s group specifies that the first category, that of isolated cells located at the periphery of the nests, have membranous/cytoplasmic staining. Unfortunately, there is no mention of the tumor site relative to the immunostaining pattern, so we do not know the immunostaining status of the five laryngeal samples their study included.

Other systems used when addressing partial p16<sup>INK4a</sup> immunostaining in HNSCC include employing a composite score based on immunostaining intensity and the percentage of positive tumor cells for cytoplasm and nucleus separately [22, 23] or based on immunostaining intensity, percentage of positive tumor cells, and confluence of immunostaining (groups of more than 10 tumor cells), both nuclear and cytoplasmic immunoreexpression needed for the cell to be considered positive [29, 30]. Out of the previously mentioned studies, only the one conducted by Zhao *et al.* included LSCC cases in the research cohort, most LSCCs belonging to a low immunostaining group.

The marginal pattern that both we and Ryu *et al.* noted has been previously described in basal cell carcinoma and squamous cell carcinoma of the skin, as p16<sup>INK4a</sup> immunoreactivity mainly seen in cells located at the edges of tumor nodules [31, 32]. Being overexpressed at the infiltrative front, it has been suggested that it is involved in tumor invasion, with a common functional role in both cutaneous tumors [17]. The growth of neoplasms might be limited by the tumor suppressive mechanism the protein is part of [33], but p16<sup>INK4a</sup>'s involvement in regulating infiltrative behavior seems to be independent of pRB, since phosphorylated pRB has been observed in the same marginal location in tumors that had p16<sup>INK4a</sup> immunoreexpression only in the cytoplasm. So, the protein was upregulated at the invasive front even though it was not exerting its function as an inhibitor of proliferation *via* the pRB pathway [34].

We found a significant association between p16<sup>INK4a</sup> immunoreexpression patterns and pN staging, with all marginal pattern cases belonging to the pNx-0 group. The result is in partial concordance with Ryu *et al.*'s [19] findings of a significant association of p16<sup>INK4a</sup> immunoreexpression patterns with pN staging; in their study, the percentage of cases showing a marginal immunoreexpression

pattern was two times higher in the pN0-1 group than in the pN2-3 one. The presence of the marginal immunostaining pattern solely in cases with no lymph node involvement suggests that p16<sup>INK4a</sup> might have a role in regulating tumor spread. Additionally, similar to the findings reported by Zhao *et al.*, we also noted that patients with the weak diffuse pattern had a higher nodal stage (pN2) [22]. On the other hand, our study found no significant differences for p16<sup>INK4a</sup> expression patterns depending on whether lymphovascular invasion was present, thus the mechanism underlying the relation between p16<sup>INK4a</sup> immunoreexpression at the periphery of tumor nests and lymph node metastasis remains an open question.

p16<sup>INK4a</sup> immunoreexpression patterns were also found to be associated with Ki67 proliferation index in our study. Tumors with p16<sup>INK4a</sup> overexpressed at the periphery of the tumor nodules had a lower Ki67 proliferation index. Through p16<sup>INK4a</sup> and Ki67 double immunostaining, Svensson *et al.* revealed ceased proliferation at the invasive front of the tumor nodules that expressed up-regulated p16<sup>INK4a</sup> at the edge of the nodules [31]. Our observation regarding the increased Ki67 proliferation index in tumors that exhibited a weak scattered or a weak diffuse immunostaining pattern is in concordance with Nilsson *et al.*'s work on cutaneous SCC, who noted an overlap between weak cytoplasmic p16<sup>INK4a</sup> immunoreexpression and presence of proliferation [34]. Also, the elevation of Ki67 proliferation index along with tumor dedifferentiation and stage that we detailed has been described by previous studies, like the one published by Ciesielska *et al.* Moreover, they revealed a moderate positive correlation between Ki67 nuclear expression and p16<sup>INK4a</sup> cytoplasmic expression in cancer cells [35].

Whereas previous studies have shown a prognostic significance of cellular localization in HNSCC [19, 22, 23], such an association was not found in our study, neither for OS nor for DFS. The apparent lack of consensus between our findings and other studies can be explained by the fact that the aforementioned studies have included cases with strong diffuse nuclear and cytoplasmic positivity in their statistical analyses, while we have only analyzed cases with equivocal staining. Also, as we pointed out earlier, previous findings refer to HNSCC as a group, including oropharyngeal tumors, and a better outcome of p16<sup>INK4a</sup> positive oropharyngeal tumors has been proven [36–38]. Low expression of CDKN2A messenger ribonucleic acid (mRNA) and p16<sup>INK4a</sup> were associated with a poor clinical prognosis independently of other known prognostic factors in HPV- HNSCC [39]. Likewise, it is believed that the overexpressed p16<sup>INK4a</sup> found in HPV+ HNSCC might be mechanistically involved in their radiosensitivity, hypothesis that has been tested by effectively radiosensitizing HPV- cells by means of CDK4/6 inhibition using Palbociclib [40]. For the minority of p16<sup>INK4a</sup> overexpressing LSCC, no prognostic significance was found even in highly homogeneous patient material, like that used by Tiefenböck-Hansson *et al.* (pT2-3N0 glottic SCC treated with radiotherapy/chemoradiotherapy) [41]. Similarly, we did not find differences related to p16<sup>INK4a</sup> staining patterns in our subgroup of patients that underwent radiotherapy/chemoradiotherapy.

Smoking-related cancers can express p16<sup>INK4a</sup>, frequently altered in function. Tobacco exposure can entail various epigenetic changes leading to aberrant p16<sup>INK4a</sup> immun-expression, with low intensity immunostaining or abnormal localization [23]. We failed to find an association between immunoexpression patterns and smoking habits. A possible explanation lies in López *et al.*'s work on a cohort of smokers with HPV- LSCC; even though almost all samples presented losses at 9p21 segment, the most common finding being a small deletion of the p16<sup>INK4a</sup> locus, overall, they found no correlation between protein expression and gene status [42].

The most significant strength of this study is the cohort consisting of a single site of HNSCC, namely the larynx. As previously mentioned, HNSCC of different subsites have been frequently grouped together. Considering the different clinical features and risk factors, this praxis needs to be reviewed, with more studies conducted on single site large cohorts. This study also has certain limitations. Because of its retrospective nature, patients are heterogeneous in stage, treatment, and other factors that might impact our findings. Also, it made quantitative assessment of tobacco exposure unachievable. Since both duration and intensity of smoking were associated with worse survival in patients with p16-positive oropharyngeal tumors [43], grouping patients as nonsmokers or current smokers and collective evaluation, is dissatisfying. Also, there are inherent limitations in the subjectivity of IHC staining interpretation due to expression heterogeneity and due to immunostaining intensity of the particular clone we used. Comparing the G175-405 clone to the E6H4 clone, both from Ventana Medical Systems, Barasch *et al.* [30] found the G175-405 clone may be more susceptible to partial reactivity, showing weaker immunostaining, especially in the nuclei of tumor cells. Final determination of p16<sup>INK4a</sup> status, however, showed concordance between the two clones. Lastly, clarifying the link between HPV infection and partial p16<sup>INK4a</sup> immunoexpression demands that HPV infection status should be evaluated. HPV status was not determined in our study, and this may be a limitation of our findings. Nevertheless, we aimed to provide insights into p16<sup>INK4a</sup> beyond its role as a immunomarker for HPV.

Human cancers have been reported to harbor mutation, promoter methylation, or deletion of the ARF/INK4a locus, with a frequency second only to *p53* gene mutations [33]. Such frequency in alterations justifies research into therapeutic approaches that could be designed around targeting p16<sup>INK4a</sup>. On one hand, therapies that restore p16<sup>INK4a</sup> by demethylation or inhibition of cytoplasmic p16<sup>INK4a</sup> sequestering proteins could lead to cancer elimination through premature senescence induction in tumors in which p16<sup>INK4a</sup> has a low expression or aberrant subcellular location [32]. On the other hand, patients with p16<sup>INK4a</sup>-overexpressing tumors, currently resistant to available compounds with specificity against p16<sup>INK4a</sup>-positive cells, could benefit from gene therapy that allows specific elimination of p16<sup>INK4a</sup>-overexpressing cells [44]. Although gene delivery is still neither safe nor feasible in humans, progress in anti-cancer interventions means that accurate knowledge of p16<sup>INK4a</sup> expression is fundamental for guiding research into new therapies.

## Conclusions

This study reaffirms that strong positivity in p16<sup>INK4a</sup> immunostaining appears in a minority of LSCC. Equivocal immunostaining shows several architectural patterns that may prove significant in stratifying characteristic clinico-pathological subgroups among LSCC, particularly referring to nodal involvement. Therefore, evaluating p16<sup>INK4a</sup> immunohistochemistry both with conventional quantitative criteria and by architectural pattern could be of value. Cytoplasmic localization of p16<sup>INK4a</sup> may represent a part of a different modulating pathway, not just a way to inactivate p16<sup>INK4a</sup>'s cell cycle control function, and might be involved in tumorigenic molecular pathways probably unrelated to HPV infection.

## Conflict of interests

The authors declare that they have no conflict of interests.

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## References

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*, 2018, 68(6):394–424. <https://doi.org/10.3322/caac.21492> PMID: 30207593. Erratum in: *CA Cancer J Clin*, 2020, 70(4):313. <https://doi.org/10.3322/caac.21609> PMID: 32767693
- Slootweg P, Grandis J. Tumours of the hypopharynx, larynx, trachea and parapharyngeal space. In: El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ (eds). World Health Organization (WHO) Classification of head and neck tumours. 4<sup>th</sup> edition, vol. 9, WHO Classification of Tumours, International Agency for Research on Cancer (IARC) Press, Lyon, France, 2017, 77–104.
- Erkul E, Yilmaz I, Nari G, Babayigit MA, Gungor A, Demirel D. The presence and prognostic significance of human papillomavirus in squamous cell carcinoma of the larynx. *Eur Arch Otorhinolaryngol*, 2017, 274(7):2921–2926. <https://doi.org/10.1007/s00405-017-4573-0> PMID: 28439692
- Matsuzaki H, Makiyama K, Suzuki H, Hirai R, Oshima T. Human papillomavirus infection status of various laryngeal diseases in Japan: a comprehensive study. *J Voice*, 2017, 31(4):504.e35–504.e40. <https://doi.org/10.1016/j.jvoice.2016.11.012> PMID: 27986378
- Mooren JJ, Gültekin SE, Straetmans JMJA, Haesevoets A, Peutz-Kootstra CJ, Huebbers CU, Dienes HP, Wieland U, Ramaekers FCS, Kremer B, Speel EJM, Klussmann JP. P16<sup>INK4a</sup> immunostaining is a strong indicator for high-risk-HPV-associated oropharyngeal carcinomas and dysplasias, but is unreliable to predict low-risk-HPV-infection in head and neck papillomas and laryngeal dysplasias. *Int J Cancer*, 2014, 134(9):2108–2117. <https://doi.org/10.1002/ijc.28534> PMID: 24127203
- Zhao R, Choi BY, Lee MH, Bode AM, Dong Z. Implications of genetic and epigenetic alterations of CDKN2A (p16<sup>INK4a</sup>) in cancer. *EBioMedicine*, 2016, 8:30–39. <https://doi.org/10.1016/j.ebiom.2016.04.017> PMID: 27428416 PMCID: PMC4919535
- LaPak KM, Burd CE. The molecular balancing act of p16<sup>INK4a</sup> in cancer and aging. *Mol Cancer Res*, 2014, 12(2):167–183. <https://doi.org/10.1158/1541-7786.MCR-13-0350> PMID: 24136988 PMCID: PMC3944093
- Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, 1993, 366(6456):704–707. <https://doi.org/10.1038/366704a0> PMID: 8259215



- [9] Xiong Y, Zhang H, Beach D. Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes Dev*, 1993, 7(8):1572–1583. <https://doi.org/10.1101/gad.7.8.1572> PMID: 8101826
- [10] Kotake Y, Naemura M, Murasaki C, Inoue Y, Okamoto H. Transcriptional regulation of the *p16* tumor suppressor gene. *Anticancer Res*, 2015, 35(8):4397–4401. PMID: 26168478
- [11] Prigge ES, Arbyn M, von Knebel Doeberitz M, Reuschenbach M. Diagnostic accuracy of p16<sup>INK4a</sup> immunohistochemistry in oropharyngeal squamous cell carcinomas: a systematic review and meta-analysis. *Int J Cancer*, 2017, 140(5):1186–1198. <https://doi.org/10.1002/ijc.30516> PMID: 27859245
- [12] Albuquerque A, Rios E, Dias CC, Nathan M. p16 immunostaining in histological grading of anal squamous intraepithelial lesions: a systematic review and meta-analysis. *Mod Pathol*, 2018, 31(7):1026–1035. <https://doi.org/10.1038/s41379-018-0026-6> PMID: 29434342
- [13] Darragh TM, Colgan TJ, Cox JT, Heller DS, Henry MR, Luff RD, McCalmont T, Nayar R, Palefsky JM, Stoler MH, Wilkinson EJ, Zaino RJ, Wilbur DC; Members of LAST Project Work Groups. The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. *Arch Pathol Lab Med*, 2012, 136(10):1266–1297. <https://doi.org/10.5858/arpa.LGT200570> PMID: 22742517
- [14] Liu Y, Alqatari M, Sultan K, Ye F, Gao D, Sigel K, Zhang D, Kalir T. Using p16 immunohistochemistry to classify morphologic cervical intraepithelial neoplasia 2: correlation of ambiguous staining patterns with HPV subtypes and clinical outcome. *Hum Pathol*, 2017, 66:144–151. <https://doi.org/10.1016/j.humpath.2017.06.014> PMID: 28705710 PMID: PMC5644341
- [15] Leeman A, Jenkins D, Marra E, van Zummeren M, Pirog EC, van de Sandt MM, van Eeden A, Schim van der Loeff MF, Doorbar J, de Vries HJC, van Kemenade FJ, Meijer CJLM, Quint WGV. Grading immunohistochemical markers p16<sup>INK4a</sup> and HPV E4 identifies productive and transforming lesions caused by low- and high-risk HPV within high-grade anal squamous intraepithelial lesions. *Br J Dermatol*, 2020, 182(4):1026–1033. <https://doi.org/10.1111/bjd.18342> PMID: 31302935 PMID: PMC7187351
- [16] McHugh D, Gil J. Senescence and aging: causes, consequences, and therapeutic avenues. *J Cell Biol*, 2018, 217(1):65–77. <https://doi.org/10.1083/jcb.201708092> PMID: 29114066 PMID: PMC5748990
- [17] D'Arcangelo D, Tinaburri L, Dellambra E. The role of p16<sup>INK4a</sup> pathway in human epidermal stem cell self-renewal, aging and cancer. *Int J Mol Sci*, 2017, 18(7):1591. <https://doi.org/10.3390/ijms18071591> PMID: 28737694 PMID: PMC5536078
- [18] Olimid DA, Florescu AM, Cernea D, Georgescu CC, Mărgăritescu C, Simionescu CE, Stepan AE. The evaluation of p16 and Ki67 immunorexpression in ameloblastomas. *Rom J Morphol Embryol*, 2014, 55(2):363–367. PMID: 24969987
- [19] Ryu HJ, Kim EK, Heo SJ, Cho BC, Kim HR, Yoon SO. Architectural patterns of p16 immunohistochemical expression associated with cancer immunity and prognosis of head and neck squamous cell carcinoma. *APMIS*, 2017, 125(11):974–984. <https://doi.org/10.1111/apm.12744> PMID: 28975663
- [20] Evangelou K, Bramis J, Peros I, Zacharatos P, Dasiou-Plakida D, Kalogeropoulos N, Asimacopoulos PJ, Kittas C, Marinos E, Gorgoulis VG. Electron microscopy evidence that cytoplasmic localization of the p16<sup>INK4a</sup> “nuclear” cyclin-dependent kinase inhibitor (CKI) in tumor cells is specific and not an artifact. A study in non-small cell lung carcinomas. *Biotech Histochem*, 2004, 79(1):5–10. <https://doi.org/10.1080/10520290310001659466> PMID: 15223748
- [21] Nilsson K, Landberg G. Subcellular localization, modification and protein complex formation of the cdk-inhibitor p16 in Rb-functional and Rb-inactivated tumor cells. *Int J Cancer*, 2006, 118(5):1120–1125. <https://doi.org/10.1002/ijc.21466> PMID: 16161044
- [22] Zhao N, Ang MK, Yin XY, Patel MR, Fritchie K, Thorne L, Muldrew KL, Hayward MC, Sun W, Wilkerson MD, Chera BS, Hackman T, Zanation AM, Grilley-Olson JE, Couch ME, Shockley WW, Weissler MC, Shores CG, Funkhouser WK, Olshan AF, Hayes DN. Different cellular p16<sup>INK4a</sup> localisation may signal different survival outcomes in head and neck cancer. *Br J Cancer*, 2012, 107(3):482–490. <https://doi.org/10.1038/bjc.2012.264> PMID: 22735904 PMID: PMC3405208
- [23] Lai S, Wenaas AE, Sandulache VC, Hartman C, Chiao E, Kramer J, Zevallos JP. Prognostic significance of p16 cellular localization in oropharyngeal squamous cell carcinoma. *Ann Clin Lab Sci*, 2016, 46(2):132–139. PMID: 27098618
- [24] Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, Loomis AM, Shah JP. Head and neck cancers – major changes in the American Joint Committee on Cancer eighth edition Cancer Staging Manual. *CA Cancer J Clin*, 2017, 67(2):122–137. <https://doi.org/10.3322/caac.21389> PMID: 28128848
- [25] Sánchez Barrueco A, González Galán F, Villacampa Aubá JM, Díaz Tapia G, Fernández Hernández S, Martín-Arriscado Arroba C, Cenjor Español C, Almodóvar Álvarez C. p16 influence on laryngeal squamous cell carcinoma relapse and survival. *Otolaryngol Head Neck Surg*, 2019, 160(6):1042–1047. <https://doi.org/10.1177/0194599818821910> PMID: 30642220
- [26] Hernandez BY, Rahman M, Lynch CF, Cozen W, Unger ER, Steinau M, Thompson T, Saber MS, Altekuse SF, Goodman MT, Powers A, Lyu C, Saraiya M. p16<sup>INK4a</sup> expression in invasive laryngeal cancer. *Papillomavirus Res*, 2016, 2:52–55. <https://doi.org/10.1016/j.pvr.2016.03.001> PMID: 29034342 PMID: PMC5637278
- [27] Young RJ, Urban D, Angel C, Corry J, Lyons B, Vallance N, Kleid S, Iseli TA, Solomon B, Rischin D. Frequency and prognostic significance of p16<sup>INK4a</sup> protein overexpression and transcriptionally active human papillomavirus infection in laryngeal squamous cell carcinoma. *Br J Cancer*, 2015, 112(6):1098–1104. <https://doi.org/10.1038/bjc.2015.59> PMID: 25688737 PMID: PMC4366899
- [28] Chen ZW, Weinreb I, Kamel-Reid S, Perez-Ordoñez B. Equivocal p16 immunostaining in squamous cell carcinoma of the head and neck: staining patterns are suggestive of HPV status. *Head Neck Pathol*, 2012, 6(4):422–429. <https://doi.org/10.1007/s12105-012-0382-3> PMID: 22801997 PMID: PMC3500888
- [29] Lewis JS Jr, Chernock RD, Ma XJ, Flanagan JJ, Luo Y, Gao G, Wang X, El-Mofty SK. Partial p16 staining in oropharyngeal squamous cell carcinoma: extent and pattern correlate with human papillomavirus RNA status. *Mod Pathol*, 2012, 25(9):1212–1220. <https://doi.org/10.1038/modpathol.2012.79> PMID: 22596101
- [30] Barasch S, Mohindra P, Henrick K, Hartig GK, Harari PM, Yang DT. Assessing p16 status of oropharyngeal squamous cell carcinoma by combined assessment of the number of cells stained and the confluence of p16 staining: a validation by clinical outcomes. *Am J Surg Pathol*, 2016, 40(9):1261–1269. <https://doi.org/10.1097/PAS.0000000000000666> PMID: 27186851 PMID: PMC4987254
- [31] Svensson S, Nilsson K, Ringberg A, Landberg G. Invade or proliferate? Two contrasting events in malignant behavior governed by p16<sup>INK4a</sup> and an intact Rb pathway illustrated by a model system of basal cell carcinoma. *Cancer Res*, 2003, 63(8):1737–1742. PMID: 12702553
- [32] Romagosa C, Simonetti S, López-Vicente L, Mazo A, Lleonat ME, Castellvi J, Ramon y Cajal S. p16<sup>INK4a</sup> overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. *Oncogene*, 2011, 30(18):2087–2097. <https://doi.org/10.1038/onc.2010.614> PMID: 21297668
- [33] Inoue K, Fry EA. Aberrant expression of p16<sup>INK4a</sup> in human cancers – a new biomarker? *Cancer Rep Rev*, 2018, 2(2):10.15761/CRR.1000145. <https://doi.org/10.15761/CRR.1000145> PMID: 29951643 PMID: PMC6018005
- [34] Nilsson K, Svensson S, Landberg G. Retinoblastoma protein function and p16<sup>INK4a</sup> expression in actinic keratosis, squamous cell carcinoma *in situ* and invasive squamous cell carcinoma of the skin and links between p16<sup>INK4a</sup> expression and infiltrative behavior. *Mod Pathol*, 2004, 17(12):1464–1474. <https://doi.org/10.1038/modpathol.3800220> PMID: 15257310
- [35] Ciesielska U, Zatonski T, Nowinska K, Ratajczak-Wielgomas K, Grzegorzka J, Piotrowska A, Olbromski M, Pula B, Podhorska-Okolow M, Dziegiel P. Expression of cell cycle-related proteins p16, p27 and Ki-67 proliferating marker in laryngeal squamous cell carcinomas and in laryngeal papillomas. *Anticancer Res*, 2017, 37(5):2407–2415. <https://doi.org/10.21873/anticancer.11580> PMID: 28476808

- [36] Ramesh PS, Devegowda D, Singh A, Thimmulappa RK. NRF2, p53, and p16: predictive biomarkers to stratify human papillomavirus associated head and neck cancer patients for de-escalation of cancer therapy. *Crit Rev Oncol Hematol*, 2020, 148:102885. <https://doi.org/10.1016/j.critrevonc.2020.102885> PMID: 32062315
- [37] Albers AE, Qian X, Kaufmann AM, Coords A. Meta analysis: HPV and p16 pattern determines survival in patients with HNSCC and identifies potential new biologic subtype. *Sci Rep*, 2017, 7(1):16715. <https://doi.org/10.1038/s41598-017-16918-w> PMID: 29196639 PMCID: PMC5711807
- [38] Brauswetter D, Birtalan E, Danos K, Kocsis A, Krenacs T, Timar J, Mihalyi R, Horcsik D, Polony G, Tamas L, Petak I. p16<sup>INK4</sup> expression is of prognostic and predictive value in oropharyngeal cancers independent of human papillomavirus status: a Hungarian study. *Eur Arch Otorhinolaryngol*, 2017, 274(4):1959–1965. <https://doi.org/10.1007/s00405-016-4412-8> PMID: 27999998
- [39] Chen WS, Bindra RS, Mo A, Hayman T, Husain Z, Contessa JN, Gaffney SG, Townsend JP, Yu JB. CDKN2A copy number loss is an independent prognostic factor in HPV-negative head and neck squamous cell carcinoma. *Front Oncol*, 2018, 8:95. <https://doi.org/10.3389/fonc.2018.00095> PMID: 29670856 PMCID: PMC5893829
- [40] Göttgens EL, Bussink J, Leszczynska KB, Peters H, Span PN, Hammond EM. Inhibition of CD4/CDK6 enhances radio-sensitivity of HPV negative head and neck squamous cell carcinomas. *Int J Radiat Oncol Biol Phys*, 2019, 105(3): 548–558. <https://doi.org/10.1016/j.ijrobp.2019.06.2531> PMID: 31271827
- [41] Tiefenböck-Hansson K, Haapaniemi A, Farnebo L, Palmgren B, Tarkkanen J, Farnebo M, Munck-Wikland E, Mäkitie A, Garvin S, Roberg K. WRAP53 $\beta$ , survivin and p16<sup>INK4a</sup> expression as potential predictors of radiotherapy/chemo-radiotherapy response in T2N0-T3N0 glottic laryngeal cancer. *Oncol Rep*, 2017, 38(4):2062–2068. <https://doi.org/10.3892/or.2017.5898> PMID: 28849066 PMCID: PMC5652956
- [42] López F, Sampedro T, Llorente JL, Hermsen M, Álvarez-Marcos C. Alterations of p14<sup>ARF</sup>, p15<sup>INK4b</sup>, and p16<sup>INK4a</sup> genes in primary laryngeal squamous cell carcinoma. *Pathol Oncol Res*, 2017, 23(1):63–71. <https://doi.org/10.1007/s12253-016-0083-4> PMID: 27377733
- [43] Grønhøj C, Jensen JS, Wagner S, Dehlendorff C, Friborg J, Andersen E, Wittekindt C, Würdemann N, Sharma SJ, Gattenlöhner S, Klusmann JP, von Buchwald C. Impact on survival of tobacco smoking for cases with oropharyngeal squamous cell carcinoma and known human papillomavirus and p16-status: a multicenter retrospective study. *Oncotarget*, 2019, 10(45):4655–4663. <https://doi.org/10.18632/oncotarget.27079> PMID: 31384393 PMCID: PMC6659794
- [44] Kohli J, Campisi J, Demaria M. A novel suicide gene therapy for the treatment of p16<sup>INK4a</sup>-overexpressing tumors. *Oncotarget*, 2017, 9(7):7274–7281. <https://doi.org/10.18632/oncotarget.23752> PMID: 29484109 PMCID: PMC5800901

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